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Comparison of the Therapeutic Effect of Sinapic Acid in Drug-Resistant and Non-Resistant Hepatocellular Carcinoma Cells

Fatma Secer Celik [1*], Canan Eroglu Gunes [2], Ercan Kurar [2]

[1] R.A.C. of Science and Technology (BITAM), Necmettin Erbakan University, Konya, Turkey
[2] Department of Medical Biology, Faculty of Medicine, Necmettin Erbakan University, Konya, Turkey

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Abstract

Sinapic acid (SA) has been shown to have anti-bacterial, anti-cancer, anti-inflammatory, cardioprotective, hepatoprotective, and neuroprotective effects in pre-clinical and *in vitro* investigations. Especially in cancer studies, it has been shown that SA has an anti-cancer effect. SA reduced proliferation and induced apoptosis in cancer cells. In this study, the effect of SA on drug resistance in hepatocellular carcinoma was investigated. For this purpose, cells resistant to sorafenib were obtained. According to the gene expression analysis performed in resistant cells, the expression of MDR1, hCE-1 and hCE-2 increased significantly. Then, specific doses of sinapic acid were applied to both Hep3B and resistant Hep3B cell lines. The IC_{50} dose for Hep3B cells was determined by XTT analysis. The cytotoxic effect of SA was different in resistant and non-resistant cells. The IC_{50} dose of SA was increased in resistant cells compared to Hep3B cells. The chemotherapeutic applications of herbal chemicals have been widely studied. However, drug resistance against frequently used chemotherapeutics is the biggest obstacle to treatment. This study showed that the dose of SA applied to resistant cells should be increased. This suggests that when the resistance mechanism occurs, the herbal chemicals are also effused out of the cell like drugs. It is necessary to study the intracellular interactions of SA with further studies.

Corresponding Author:
Fatma Secer Celik, R.A.C. of Science and Technology (BITAM), Necmettin Erbakan University, Konya, Turkey, bio.secer@gmail.com
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1. INTRODUCTION

All around the world, there has been a noticeable increase in the use of plant-based goods. Numerous potential benefits for treating conditions like cancer, diabetes, cardiovascular diseases and many others are claimed for the phytochemicals that may be found in plants (Robbins, 2003). Previous studies have shown that phytochemicals derived from plants are typically non-toxic when ingested in smaller amounts and that they carry out various biological functions (Soobrattee *et al*., 2005). Because they are thought to be more efficient and safer than synthetic drugs, the use of herbal medicines and nutraceuticals has steadily increased around the world (Ekor, 2014).

One of the most prevalent hydroxycinnamic acids, sinapic acid (SA), is found across the plant kingdom (Chen, 2016). As valuable sources of SA, spices, oil seeds, vegetables, citrus fruits, and cereals are noted. Additionally, SA has reportedly been identified as a key ingredient in many traditional Chinese medicines (Menezes *et al*., 2011).

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SA, 3,5-dimethoxy-4-hydroxycinnamic acid chemically, may be found both in its free form and as esters (Nićiforović and Abramovič, 2014). SA has the ability to combine with ferulic acid to produce dimers (Kern *et al*., 2003). It is a crystalline powder coloring yellow-brown and has a molecular weight of 224.21 g/mol. SA is easily soluble in DMSO and carbitol, but not effectively soluble in water (Alshahrani and Shakeel, 2020). Despite the fact that there are few studies indicating SA's toxicity, certain studies have examined SA's toxicity in various models. Studies employing the superoxide-scavenging test revealed that SA's cytotoxic activity was somewhat higher than that of its ester derivate (Chapple *et al*., 1992).

SA is known as a significant chain-breaking antioxidant with effective scavenging abilities (Lee-Manion *et al*., 2009). The ability of SA to donate hydrogen atoms and its capacity to use the conjugated system to stabilize the resultant phenoxyl radicals are related to its action as an antioxidant (Cos *et al*., 2002). Additionally, according to reports, SA performs an antioxidant function that is significantly more important than that of ferulic acid and caffeic acid (Teixeira *et al*., 2013).

The anti-cancer properties of SA were investigated in PC-3 and LNCaP human prostate cancer cell lines (Eroğlu *et al*., 2018). SA doses ranging from 250 µM to 3000 µM were employed and IC_{50} value was reported to be 1000 µM. In PC-3 cells, SA significantly elevated expressions for the BCL2 associated X (Bax), caspase-3 (CAS3), caspase-7 (CAS7), cytochrome c (CYCS), Fas cell surface death receptor (FAS), TIMP metallopeptidase inhibitor 1 (TIMP-1) and cadherin 1 (CDH-1) genes, but downregulated gene expressions of cadherin 2 (CDH2), matrix metallopeptidase 2 (MMP-2), and matrix metallopeptidase 2 (MMP-9). In PC-3 and LNCaP cells colorimetric analysis of caspase-3 activity revealed 7.81 and 4.04 fold increases on SA-treated groups, respectively. SA treatment also decreased cell invasion of PC3 (33.3%) and LNCaP (27.27%) cells (Eroğlu *et al.* 2018).

Previous study also reported that SA has antibacterial features against the phytopathogen *Xylella fastidiosa*. According to the agar disc-diffusion test and numerous other comparable analysis, SA was discovered to have a substantial anti-bacterial action together with the other plant secondary metabolites (Maddox *et al*., 2010).

Drug resistance is a complicated phenomenon that typically arises from chemotherapy inability to treat cancer. Through numerous mechanisms and pathways, malignant cells increasingly develop resistance to various chemotherapy treatments. Understanding the molecular processes underlying drug resistance continues to be a crucial field of study for pinpointing targets and developing new medications to enhance therapeutic effects.

Multi drug resistance (MDR) and single drug resistance are controlled by intricate mechanisms, some of which use traditional methods. Anti-cancer drug-induced regulation of apoptosis and autophagy in cancer cells is one mechanism of MDR (Matuszcak *et al*., 2014). There are currently 49 members of the ATP-binding cassette (ABC) superfamily, which has been subdivided into 7 subfamilies (ABCA to ABCG) based on structural similarities (Chang and Roth, 2001). Of these, ABCB1 (MDR1) is the most important due to its unique function in chemoresistance. Breast cancers, pediatric tumors, acute myeloid leukemia and hematological malignancies have all been associated with MDR1 overexpression, which can be controlled by tumor-related signaling such PI3K/Akt signaling (Tazzari *et al*., 2007; Burris, 2013). Chemo drug has been described as one of the primary inducers of MDR1 (Hu *et al*., 1995; Zhou and Ling, 2010; Housman *et al*., 2014). The aim of this study was to investigate the cytotoxic effect of sinapic acid in drug resistant cells compared to normal cancer cells.

2. MATERIAL and METHODS

2.1. Chemicals

SA was purchased commercially from the Sigma-Aldrich Chemical Company. XTT [2,3bis(2Methoxy4nitro5sulfophenyl) 2H tetrazolium5carboxanilide] kit was purchased from Biological Industries. The TRIzol reagent was obtained from Invitrogen. A commercially available Transcriptor First Strand cDNA Synthesis Kit was made available by Bio-Rad.

2.2. Cell Culture

Hep3B (ATCC® HB-8064™) hepatocellular carcinoma cell line was obtained from ATCC. These cells were grown in EMEM (Eagle's Minimum Essential Medium) medium with 10% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine at 37 °C in a cell culture incubator under a proliferating 5% CO_2 environment. The cells were used in the subsequent studies after confluence reached 80%.

2.3. Generating Drug Resistant Hep3B Cell Culture

Hep3B cells were grown in EMEM medium in T25 flasks. Then, it was inserted into six-well plates. Applications of sorafenib began at 2 µM. Depending on the state of the cell's growth, the drug dose was increased by 0,25 µM every two weeks. Cellular RNA was obtained four months later, and expression of drug-resistance genes were analyzed.

2.4. Cell Proliferation Analysis

The cytotoxic effect SA and IC_{50} dose was assessed in Hep3B cells using the XTT test. SA was dissolved in dimethyl sulfoxide (DMSO). In a 96-well plate, $2x10^3$ cells were planted per well, and the cells were then incubated for 24 hours. Different doses of SA (250 µM, 500 µM, 750 µM, 1000 µM, 1500 µM, 2000 µM, 2500 µM and 3000 µM) were applied to cells for 24, 48 and 72 hours. Plates were incubated again for 4 hours after 50 µl of XTT solution had been added to the wells. Then, using a microplate reader, absorbance at 450 nm (the reference wavelength) was used to calculate the amount of cell viability.

2.5. RNA Isolation, cDNA Synthesis and qPCR Analysis

TRIzol Reagent was used to carry out the total RNA isolation using the protocol suggested by manufacturer. For DNA contamination, applied Dnase to total RNA. cDNA synthesis was carried out using the Transcriptor First Strand cDNA Synthesis Kit. An online IDT PrimerQuest (https://eu.idtdna.com/site) program was used to design the target gene primer sequences (Table 1) used in the qPCR. A 10 µl final volume qPCR mix including 5 µl EvaGreen Supermix, 5 pMol of each primer and 2 µl cDNA was prepared. The PCR profile was made up of 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The initial denaturation took place at 95 °C for 10 min.

Table 1. Primer sequences of genes used in this study

No	Gene	Forward Primer (5'->3')	Reverse Primer (5'->3')	bp
1	MDR1	CCCATCATTGCAATAGCAGG	TGTTCAAACTTCTGCTCCTGA	147
2	hCE-1	AGAGGAGCTCTTGGAGACGACAT	ACTCCTGCTTGTTAATTCCGACC	89
3	hCE-2	AACCTGTCTGCCTGTGACCAAGT	ACATCAGCAGCGTTAACATTTTCTG	166
4	GAPDH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA	307

2.6. Statistical Analysis

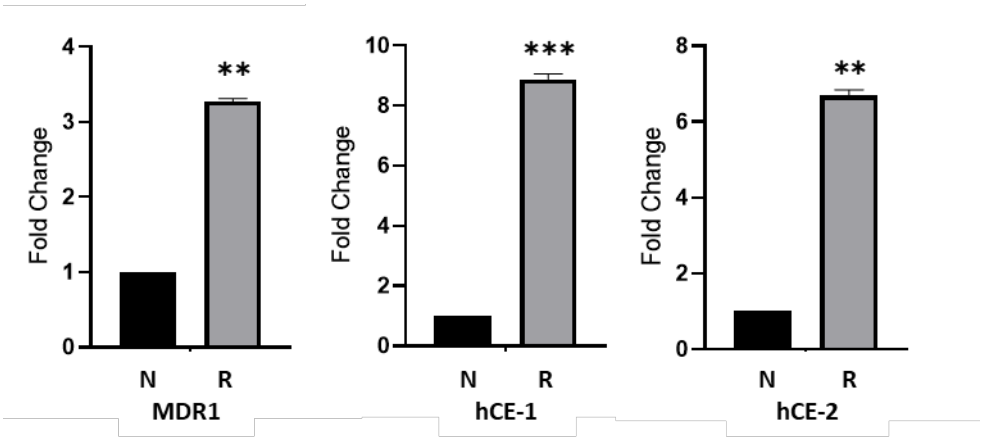
All experiments were repeated three times and are displayed as mean ± SD (standard deviation). The $2^{-\Delta\Delta Ct}$ technique was used to conduct the RT-qPCR study. The statistical evaluation was conducted using the RT^{2}Profiles^{TM}PCR Array Data Analysis using the "student *t*-test," which compares the differences between the groups.

3. RESULTS and DISCUSSION

3.1. Resistant Hep3B Cells Showed Changes in Genes Associated to Drug Resistance

Hep3B cells gained resistance by increasing the dose of sorafenib applied. Sorafenib administration was started with 2 µM, the dose was increased by 0.25 µM every 2-3 passages. Finally, it was shown by gene expression analysis that resistance occurred at 3 µM. After approximately four months of first treatment, MDR1 gene expression in cells was analyzed. In addition, expression changes in carboxylesterase genes were also investigated. According to the results, a significant increase in MDR1, hCE-1 and hCE-2 genes were observed in sorafenib administered cells (Figure 1).

Figure 1. Expression changes in MDR1 and carboxylesterase enzyme genes (N: Normal, R: resistant Hep3B cells, ***p*>0.05, ****p*>0.005)



Chemicals with a functional group, such as a carboxylic acid ester, amide or thioester, are hydrolyzed by carboxylesterases (CESs; hCE-1 and hCE-2). Some CESs also catalyze synthesis and transesterification processes in addition to hydrolysis. Carboxylesterases are important in the conversion of many cancer drugs. The anticancer prodrug capecitabine is converted by CES1 to 5′-deoxy-5-fluorocytidine (Tabata *et al*., 2004). By hydrolyzing CPT-11 (irinotecan), a topoisomerase I toxin, CES2 produces the active anticancer medication SN-38 (Humerickhouse *et al*., 2000). Significant increase in gene expressions of MDR1, hCE-1 and hCE-2 after sorafenib resistance showed that it is effective in efflux mediated drug resistance and metabolizing sorafenib in Hep3B cells. In this study, the increase in the expression of all three genes clearly shows that drug resistance occurred.

3.2. Sinapic Acid Showed Cytotoxic Effect in Hep3B Cells

It has been shown that SA is toxic to cancer cells (Balaji *et al*., 2014; Janakiraman *et al*., 2015). However, its effect on cancer cells that have gained drug resistance has not been fully elucidated yet. In this study, SA has been shown to have a cytotoxic effect in non-resistant liver cancer cells. However, it was determined that the IC_{50} dose increased significantly in resistant liver cancer cells compared to normal cancer cells (Figure 2 and Figure 3). The reason for this can be explained as reducing the intracellular effect of the drug efflux mediated multi drug resistance mechanism.

Figure 2. The cytotoxic effects of SA on Hep3B cells.

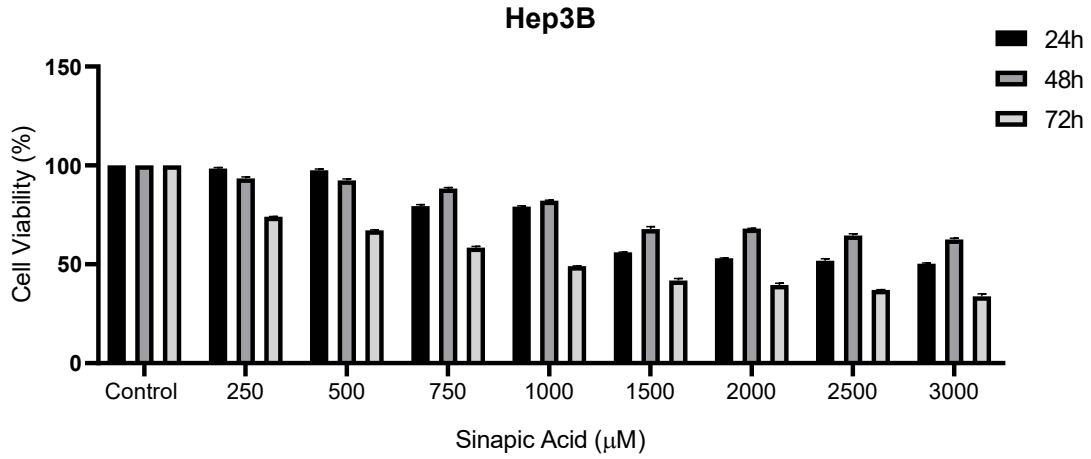
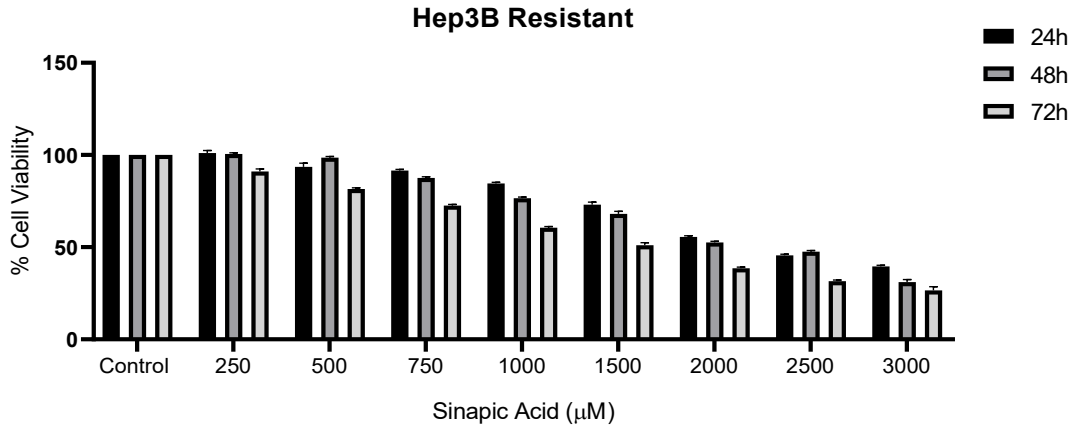


Figure 3. The cytotoxic effects of SA on resistant Hep3B cells.



According to the cell proliferation analysis, the IC_{50} dose of SA in Hep3B cells was calculated using an online software (https://www.aatbio.com/tools/ic50-calculator). The IC_{50} values of sinapic acid in the normal cells were similar to our previous study (Eroğlu *et al*., 2017) and resistant cells obtained in the current study are shown in Table 2.

Table 2. IC_{50} doses of Hep3B and Hep3B resistant cells

	IC_{50} doses (µM)	
	Hep3B	Hep3B Resistant
24h	989,13	2667
48h	1000,5	2059,5
72h	708,1	1591

About 30% of individuals with early-stage breast cancer will develop recurrent disease, despite the fact that systemic drugs used to treat cancer (for instance; cytotoxic, hormonal and immunotherapeutic agents) are typically active at the start of therapy (for example; 90% of primary breast tumors and 50% of metastases). Resistance to therapy is not just frequent; it is anticipated (Gonzalez-Angulo *et al*., 2007). Chemotherapeutic drug resistance becomes more

obvious in tumor cells from recurrent tumors (Li *et al*., 2014), and exposure to chemotherapeutic drugs can encourage the development of drug resistance in tumor cells, which ultimately results in the failure of the chemotherapeutic treatment (Ghandadi *et al*., 2016), severely restricting the range of therapeutic modalities that are effective.

4. CONCLUSION

Most cancer patients have residual tumor cells found after therapy, and it is believed that these cells can lay latent for years before resuming growth and causing tumor recurrence. Therefore, understanding the molecular mechanisms underlying this acquired cellular resistance is essential for predicting tumor resistance and enabling the development of novel therapies. Many phytochemical drugs are under investigation, but their effect on resistance or relapsing tissues is ignored. In this study, we showed that sinapic acid would be cytotoxic to cancer cells when administered at a higher dose.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Fatma Secer Celik: Investigation, methodology, writing. **Canan Eroglu Gunes:** Methodology, review, editing. **Ercan Kurar:** Review, editing, methodology.

Orcid

Fatma Secer Celik https://orcid.org/0000-0001-5619-8958
Canan Eroglu Gunes https://orcid.org/0000-0002-3796-575X
Ercan Kurar https://orcid.org/0000-0002-9234-1560

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Conservation in Tissue Culture of *Malacocarpus crithmifolius* (Retz.) Fisch. & C.A.Mey. - Relict Species from Mangyshlak

Valentina Mursaliyeva [1*], Balaussa Sarsenbek [1], Tlek Mukhanov [1], Akjunus Imanbayeva [2]

[1] Institute of Plant Biology and Biotechnology, Almaty, Kazakhstan
[2] Mangyshlak Experimental Botanical Garden, Aktau, Kazakhstan

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Abstract

Malacocarpus crithmifolius is an endangered rare relict with small size populations grown in a limited area. The purpose of this study was to initiate *in vitro* and micro replication of *Malacocarpus crithmifolius*. The vegetative and reproductive explants and Murashige & Skoog medium (MS) supplemented with different concentrations and combinations of cytokinin and auxins were used. The response reaction of different explants and the plant growth regulators' effects on shooting frequency, the number of shoots, callusogenesis, and rooting percent were investigated. Culturing generative explants on the MS medium containing 6-Benzylaminopurine (BAP) combined with indole-3-acetic acid (IAA) or indole-butyric acid (IBA) led to direct shoot regeneration. The bud mass appeared in the leaf axil under inflorescences after one week of cultivation on the initiated medium. The replication was reached by micro-cutting of induced additional shoots and 3-fold passaging them on the fresh mediums. The average multiplication result at MS with BAP in combination with 1-Naphthaleneacetic acid (NAA) or IAA composed 5.85 after three months of cultivation. In conclusion, the applicable explant and phytohormonal composition of nutrient medium for initiation and micropropagation of *M. crithmifolius* were developed.

Corresponding Author:
Valentina Mursaliyeva, Institute of Plant Biology and Biotechnology, Almaty, Kazakhstan, gen_mursal@mail.ru
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1. INTRODUCTION

The position of Kazakhstan in the center of the Euro-Asia continent specifies its rich biological diversity. According to bio-geographical land area characteristics (Takhtadzhian *et al*., 1986), most of the Kazakhstan territory is related to the Iran-Turan floristic region of the Mediterranean Subkingdom. A brief review of Kazakhstan flora biodiversity has been presented in the reviews (Ryabushkina *et al.,* 2008; Gemedjieva *et al.*, 2010).

The Mangyshlak Peninsula is located in the desert zone of Western Kazakhstan and geologically is a western part of the Ustyurt Plateau. This oil and gas field development region is characterized by difficult physical and geographical conditions: a sharply continental arid climate, salinity, tension of the wind, and a variety of landscape and climatic zones (Dzhanalieva *et al*., 1998). Mainly these factors determine a very low rate of plant introduction and an increase in the number of threatened species (Baytulin, 2010). According to a botanical study, the unique flora of Mangyshlak is represented by 675 species with the prevailing of xerophytes (Imanbayeva *et al.*, 2017). The invaluable genetic resources of endemic species are included in the Red Book of Kazakhstan (Baytulin and Sitpaeva, 2014).

Malacocarpus crithmifolius (Retz.) C.A. Mey is a relict representative of the oldest monotypic Iranian-South Turonian genus *Malacocarpus* which belongs to the family

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Nitrariaceae (Pavlov, 1963). The total area of species includes northern Iran, Afghanistan, and Middle Asia, but the main natural populations of *M. crithmifolius* are situated on the eastern seaside of the Caspian Sea: Mangyshlak and the Buzachi Peninsula (Belousova *et al.,*1979).

M. crithmifolius is a foodstuff and decorative shrub with spread-out branches, soft pinnately split leaves, lemon-yellow color flowers, and fruit red succulent three hides' berry. It is the poorly competitive species occurring in small quantities in a restricted area that can disappear by an unfavorable change in the environment and anthropogenic pressure. Since 2008 *M. crithmifolius* is presented in the Red List of the International Union for Conservation of Nature (IUCN) as a category III (VU) vulnerable species with low population size and limited range. The evaluation of the modern status of rare species (Shomurodov *et al.,* 2015) shown that revealed three populations of *M. crithmifolius* were characterized as aging and incomplete due to the absence of the generative fraction.

M. crithmifolius as a drought and salt-tolerant plant is recommended for widespread use on salted inconvenient lands and introduction in the industrial areas of Mangyshlak and Ustyurt, and the desert regions of Central Asia (Arzumanov *et al.,* 2015). The biochemical composition and biological activities of *M. crithmifolius* were studied insufficiently and the available information is limited by a few data. The juicy orange-red berries contain a lot of vitamin C, sugars, and organic acids (Belousova *et al.,* 1979; Sokolov, 1988). The high amount of alkaloids in the leaves is revealed (Zharekeev *et al,* 1971).

For the protection and conservation of endangered taxon, tissue culture techniques and micropropagation are widely used as alternative methods (Fay, 1992; Johnson, 2002). The study aims to evaluate tissue culture responses of different explants, to initiate *in vitro* and micro replication for the conservation of *M. crithmifolius*.

2. MATERIAL and METHODS

2.1. Plant Material

The initial material of *M. crithmifolius* for research purposes was collected from natural populations on the territories of the Mangyshlak Peninsula. The identification of the plants was made according to Pavlov (1963). The voucher specimens are deposited in the herbarium of the Mangyshlak Experimental Botanical Garden (MEBG).

The mature fruits were obtained in early autumn September from the MEBG. The small seeds long 3 mm were removed immediately from brownish-red berries and were used in studies (Figure 1A, Figure 1B). The seeds were planted in the greenhouse to obtain donor plants, that were transferred to the field for vegetation and flowering (Figure 1C, Figure 1D).

The leaves, vegetative shoot tip with an apical bud, and segment of the generative shoot with inflorescences isolated from flowering field-grown plants were used as explants *in vitro*. After cutting the collected material was washed thoroughly with running water. In the laminar air cabin, the shoot segments were dipped in 70% ethanol, followed by sterilization in a 0.1% mercuric chloride solution for 8 min. The explants were finally washed thrice with sterilized water and were trimmed to 1 cm long segments before its transferred to jars with 30 ml of a solidified culture medium.

Figure 1. Initial plant material of *M. crithmifolius*: berries (A), seeds (B), seedling (C), flowering plant (D).

2.2. Seed Germination *in vitro*

At first, the mature seeds isolated from half-dry fruits were an immersion in water at night for the seed cover softening. The seeds after sterilization by 0.1% solution mercuric chloride for 10 min were inoculated on Knop's medium supplemented with 20 gL^{-1} sucrose for the emergence of the seedling. The cultures were grown at 25±2ºC in a light culture room.

2.3. Nutrient Medium and Culture Conditions

Murashige & Skoog salts (1962) with 3% sucrose and 0,6% agar bacteriology grade (AppliChem, Germany) were used to prepare the medium with pH to 5,8 adjusted before autoclaving at 121ºC for 20 min.

The basic MS medium was supplemented with plant growth regulators (PRG): to initiate shoot regeneration - 4 mgL^{-1} BAP + 0.25 mgL^{-1} NAA and 1.5 mgL^{-1} BAP + 0.1 mgL^{-1} IAA; for callus tissue induction - 1 mgL^{-1} 2,4-D + 0.5 mgL^{-1} BAP; 1 mgL^{-1} 2,4 D; for shoot rooting induction – half strength (½ MS) containing 0.5 mgL^{-1} indole-butyric acid (IBA).

Five replicated (culture jars) and ten explants in each replicate were used. All cultures were maintained at 25±2ºC in the culture room with a 16/8h light/dark photoperiod under cool white fluorescent light. The cultures were transferred to freshly prepared medium monthly.

The frequency of shoot formation, the number of regenerated shoots, the callus induction percentage, and shoot rooting *in vitro* were recorded weekly. The multiplication rate (Mr) as the total number of formed shoots in the variant, divided by the number of primary explants or passaged microshoot was defined.

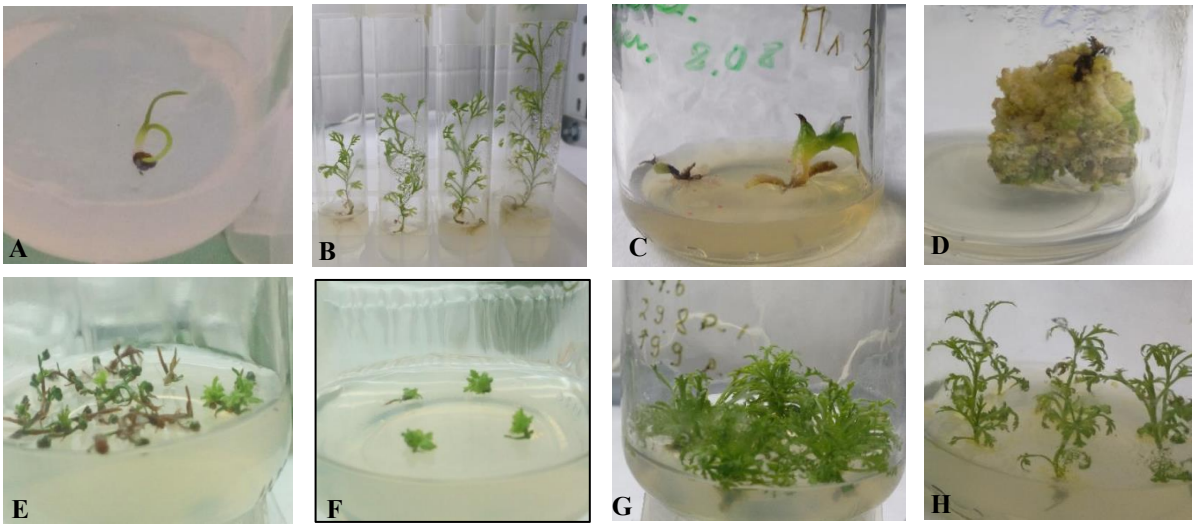
All the experiments were twice replicated with five culture tubers in each treatment. The means were employed using Microsoft Excel 2010. Differences were considered statistically significant at $p \leq 0.05$.

3. RESULTS and DISCUSSION

3.1. Seed Germination *in vitro*

It was established that the seeds *M. crithmifolius* immediately removing from ripe fruits possess high germination on the Knop's medium *in vitro* culture. The maximum germinates energy was reached in the 1st culture's week. The seedling cotyledonary leaves on the 4st inoculation day were developed (Figure 2A). The obtained seedlings were significantly varied in the length range 4-12 cm by the end of one-month cultivation (Figure 2B).

Figure 2. *M. crithmifolius* in vitro: seed germination on 4st day (A), one-month seedlings (B), shoot tip on MS + 1.5 mgL^{-1} BAP (C), callus on MS + 1 mgL^{-1} 2.4 D (D), shoot bud regeneration on MS + 4 mgL^{-1} BAP + 0.25 mgL^{-1} NAA (E), bud's conglomerates, I sub-culture (F), regenerative shoots, 3 sub-culture (G), shoot rooting on ½ MS + 0.5 mgL^{-1} IBA (H).



The obtained data indicate the seeds do not require a long ripening period after harvest and they are characterized by synchronous and friendly germination without the use of additional treatments. Moreover, the seedlings show significant differences in morphological parameters, which is typical for seed reproduction.

3.2. Vegetative Explants Responses

The leaf segments did not react *in vitro* and necrosed after two-week cultivation on all variants of MS medium. It should be noted that the leaves are bare, alternative, irregularly cleaved on lanceolate-linear lobes.

The viability of shoot tip explants was not above 30 %. The explant adaptation process *in vitro* took a long period and did not have regenerative results during further inoculation on fresh medium. The responsible reaction was promoted strongly in all media formulations but in different morphogenic responses. A significant influence of the growth regulators in the MS medium on the explant response was revealed.

The shoot explants cultured on MS supplemented with BAP had the character vitrification traits: the thickened watery leaves and shoots. During cultivation, the generative explants lost their original morphology and ability to further grow (Figure 2C).

MS containing 2 mgL^{-1} 2.4 D induced callus on the whole surface of the explant. The callus tissue mostly appeared on 12-15 culture days with high frequency. The arising callus was green in color, compact, and with a dense structure after one month of cultivation (Figure 2D). For the induction of adventitious shoot buds, the callus was sub-cultivated on MS with BAP at concentrations of 0.1 mgL^{-1} and 1 mgL^{-1}. The callus did not possess regeneration potencies and was necrotized during further cultivation. Further optimization of the hormonal composition of the medium is necessary for organogenesis induction.

3.3. Shoot Regeneration from the Generative Explant

The survival of explants isolated from generative shoots was high, about 70 %. The medium supplement by cytokinin BAP induced morphogenetic reaction of the generative explants. The cultivation in the MS with BAP in auxin combination led to the bud differentiation on a mean of 35.8 % explants. The bud conglomerate appeared in the leaf axil under inflorescences after one week of cultivation on MS 4 mgL^{-1} BAP + 0.25 mgL^{-1} NAA (Figure 2E). The induced buds were separated and transformed on fresh medium with a reduced concentration of BAP 1 mgL^{-1} for elongation of adventitious shoots (Figure 2F). It allowed us to separate about 5 single shoots from one dense inoculant. The remaining conglomerate was divided into separate pieces for the next sub-cultivation on the fresh medium for differentiation and elongation of new shoots. After twice subcultures, regenerative shoots increased in size and reached a height of 3 cm in 2 months (Figure 2G) In general, from one responsible primary explant, nearly 23 - 36 adventitious shoots depending on the growth regulators of the medium over 4 passages were obtained (Table 1).

Table 1. The effect of PGR treatment on the shoot initiation percentage, the mean number of shoot regenerated on passages, and the multiplication rate of *M. crithmifolius* measured 12 weeks after culture.

Plant growth regulators (PGR,mgL^{-1})	Bud initiation % 0 passage	Mean regenerated shoots number during passage			The multiplication rate (Mr)
		I	II	III	
4 BAP + 0.25 NAA	31.5	5.3 ± 0.6 a	8.5 ± 0.9 a	35.6 ± 4.3 a	6.7
1.5 BAP + 0.1 IAA	40.2	4.6 ± 0.4 a	3.6 ± 0.2 b	23.0 ± 4.1 b	5

As shown in Table 1, a significant effect of PRG on the number of regenerated shoots during sub-cultivation was established. The mean regenerated shoots per explant were significantly higher at MS with higher cytokinin concentration - 4 mgL^{-1} BAP + 0.25 mgL^{-1} NAA. By the end of the third passage after initiation *in vitro* the whole multiplication rate Mr was 6.7 at MS with 4 mgL^{-1} BAP + 0.25 mgL^{-1} NAA and the value was decreased in 1.3 at 1.5 mgL^{-1} BAP + 0.1 mgL^{-1} IAA.

The root development at 12.5 % aseptic shoots was noted by the first culture week on induced medium ½ MS with 0.5 mgL^{-1} IBA (Figure 2H). The data increased to 37.5 % in the second week and by the end of the monthly cycle, rhizogenesis is observed in all passivated shoots.

Thereby, the first study concerning on establishment of *in vitro* cultures and micropropagation of *M. crithmifolius* was conducted. This study revealed a high regenerative potential of the relict species. Using the suitable generative explant and optimized medium by plant growth regulators are the two main bases for the micropropagation of *M. crithmifolius*. Data on the callusogenesis process can be useful in molecular-genetic transformation studies and for using the system *in vitro* as a feasible source for obtaining plant secondary metabolites.

4. CONCLUSION

In this study, it can be concluded that there is the possibility to apply culture *in vitro* regeneration for preservation and the replication of *M. crithmifolius* that allows preventing the disappearance of the genetic resources of the threatened plant. The node segment of the generative shoot is an optimal explant for successful direct shoot regeneration on induced medium supplemented by cytokinin BAP and auxin NAA. The effective shoot rooting on ½ MS medium with 0.5 mgL^{-1} IBA at one-month cultivation is achieved. The developed technique will help in mass reproduction for the further economic use of *M. crithmifolius* (for landscaping, in garden and park construction, in vegetative reclamation measures, etc.) in the harsh soil and climatic conditions of the desert Caspian zone.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Valentina Mursaliyeva: Conceptualization, methodology, writing, editing, validation**. Balaussa Sarsenbek:** Experimental design, laboratory work, **Tlek Mukhanov:** Field work, statist analysis. **Akjunus Imanbayeva:** Resources, plant material, project administration.

Orcid

Valentina Mursaliyeva https://orcid.org/0000-0003-4803-9169
Balaussa Sarsenbek https://orcid.org/0000-0002-7951-4586
Tlek Mukhanov https://orcid.org/0000-0002-7590-769X
Akjunus Imanbayeva https://orcid.org/0000-0003-0101-6840

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Investigation of *Viburnum opulus* L. Apoptotic Effect on LNCaP Prostate Cancer Cell Line

Omer Gok [1], Fatma Secer Celik [2], Canan Eroglu Gunes [2], Ercan Kurar [2*]

[1] Faculty of Medicine, Necmettin Erbakan University, Konya, Turkey
[2] Department of Medical Biology, Faculty of Medicine, Necmettin Erbakan University, Konya, Turkey

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Abstract

The most common cancer seen in men worldwide is prostate cancer. It ranks second in the deadliest cancer types. *Viburnum opulus* is a type of tree that produces small red fruits. Shell, fruit, flower and juice of *V. opulus* are widely used in various fields and traditional medicine. Studies have shown that *V. opulus* also has an anti-cancer effect. For this purpose, the apoptotic effect of *V. opulus* was investigated in the LNCaP prostate cancer cell line. LNCaP human prostate cancer cells were treated with *V. opulus* extract in a 1-200 µg/mL concentration range. IC_{50} dose of *V. opulus* extract was determined by cell viability test XTT. Expressions of genes related to apoptosis after extract application were determined by quantitative RT-PCR analysis. *V. opulus* showed the cytotoxic effect on the LNCaP cell line, and differences in the expressions of apoptosis-related genes were observed. Significant increases in BAX, CASP7, CASP8 and P53 expressions showed that the pro-apoptosis pathway was active, while CASP3 expression was significantly decreased. The extrinsic apoptosis pathway of might be effective due to increased CASP7 and CASP8 gene expression. However, the molecular mechanism of apoptotic activity of *V. opulus* needs to be investigated in more detail.

Corresponding Author:
Ercan Kurar, Department of Medical Biology, Faculty of Medicine, Necmettin Erbakan University, Konya, Turkey, ekurar@erbakan.edu.tr
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1. INTRODUCTION

Prostate cancer is the most common type of cancer in men and ranks second after lung cancer in cancer-related deaths (Siegel *et al*., 2019). Ethnic group, age, diet and heredity are risk factors determined for this disease (Szliszka *et al*., 2011). Prostate cancer treatment options are limited due to late diagnosis, poor drug tolerance, and multiple drug resistance (Johnson *et al*., 2017). The molecular mechanisms responsible for the onset and progression of prostate cancer have not yet been fully elucidated.

Apoptosis-dependent mechanisms are often used for cancer treatment and regression of progressive cancers. Cancer cells are exposed to stress or a number of environmental agents to increase apoptotic gene expression, thereby dragging the cell to apoptosis. The regression mechanism of cancer is mainly apoptosis dependent and it is essential to illuminate the external factors and substances that induce the apoptotic pathway and cause the cells to drift into apoptosis. *In vitro* studies showed that some herbal extracts induce apoptosis in prostate cancer cells (Deng *et al*., 2017; Turan *et al*., 2017).

Viburnum opulus L., called gilaburu in Turkey, is a red-colored, chickpea-sized tree from the Viburnaceae family. It shows a wide distribution from South America to Southeast Asia and is also cultivated in Kayseri and surrounding areas of Turkey. Shell, fruit, flower and

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juice of *V. opulus* are widely used in traditional medicine and in various fields. In Asia and Europe, *V. opulus* is used for cough treatment, colds, shortness of breath, high blood pressure, tuberculosis, stomach pain, digestive system disorders, duodenal ulcers, infections and bleeding of the urinary system (Kraujalyt *et al*., 2012). *V. opulus* has also urine-enhancing, laxative and soothing effects. In Turkey, oral consumption of *V. opulus* juice is widely used for alternative therapy and prevention of kidney stones. The juice of the gilaburu fruit is often used against bile and liver diseases in the Central Anatolian region (Sezik *et al*., 2001). Fruits are also consumed as nuts (Baytop, 1999). Several compounds were determined in *V. opulus* juice such as intensely chlorogenic acid (798.81+ 6.49 mg/L), caffeic acid, p-coumaric acid, p-hydroxybenzoic acid and myricetin compounds (Çam, 2005). Previous studies reported that *V. opulus* have spasmolytic (Cometa *et al*., 2009), hepatoprotective, hypoglycemic (Sever Yılmaz *et al*., 2006), antioxidant, anticholinesterase (Orhan *et al*., 2011) relaxed, anti-inflammatory and potential anticarcinogenic effect (Kajszczak *et al*., 2020).

Prostate cancer is also a significant disease in terms of chemopreventive strategies due to its late onset age, slow progression, high incidence, identifiable preneoplastic lesions and risk groups. In developed countries, different organic compounds are preferred as supportive therapy in the conservative treatment of prostate cancer including postoperative radiotherapy and chemotherapy. For this purpose, it is critical to study the effect of the extracts and compounds obtained from different natural sources on cancer cells, especially at the molecular level. These studies can potentially contribute to the development of alternative methods for understanding and treating cancer. Therefore, this study aimed to investigate the cytotoxic effect of *V. opulus* extract and expression levels of marker genes in the apoptosis pathway in the LNCaP prostate cancer cell line.

2. MATERIAL and METHODS

2.1. Chemicals

The active substance was obtained from the Hawaiian Farm Company (USA). It was diluted in water-based cell medium.

2.2. Cell Culture

The proliferation of LNCaP (ATCC® CRL1740™) human prostate cancer cells was provided in an appropriate culture medium using RPMI-1640 (Biological Industries), 10% fetal bovine serum, 2 mM L-glutamine, 1% Penicillin-Streptomycin. The cells' proliferation, passages, and follow-up of were monitored with an inverted microscope and incubated at 95% humidity and 5% CO_2 until sufficient reproduction was achieved.

2.3. Cell Viability Assay

Cell proliferation assay with XTT Reagent-Cell Proliferation Kit (Biological Industries) was conducted. The kit contains "XTT" tetrazolium salt as a reagent. The studied groups' absorbance values (OD) were read in ELISA at 450 nm wavelength and in the reference range of 480-630 nm after 4 hours from adding XTT solution to the pre-cultured cells in 96-well plate. Cell viability ratio (%) was calculated by dividing the optical density value measured in each well by the control optical density value.

2.4. Total RNA Isolation, cDNA Synthesis and qRT-PCR Analysis

Total RNA isolation was performed from LNCaP cells for mRNA-level expression studies. For this purpose, 1 mL of TRIzol was added to the cells planted in 6-well plates. Homogenate was placed in eppendorf tubes. After incubation for 10 minutes at room temperature, 200 µl of chloroform was added to each eppendorf tube and pipetted again then incubated for 15 minutes at room temperature. Then, it was centrifuged at 15,000 g for 20

minutes at +4 ºC and the supernatant was collected and taken into separate eppendorf tubes. Five hundred µl of isopropanol was added and kept at room temperature for 10 minutes. After centrifuging at 15,000 g for 30 minutes at +4 ºC, the supernatant was carefully discarded, and the pellet was washed using 70% ethanol. After centrifugation for 10 minutes at +4 ºC, the pellet was dried briefly and dissolved with 30 µl nuclease-free water. The quality and quantity of total RNAs isolated from the control and dose groups were measured by using Nanodrop at 260/280 nm UV and agarose gel electrophoresis technique.

Table 1. Primer pairs used in the qPCR analysis.

Genes	Primer sequences	PCR product length (bp)
BAX	F: GGAGCTGCAGAGGATGATTG	151
	R: GGCCTTGAGCACCAGTTT	
BCL2	F: GTGGATGACTGAGTACCTGAAC	125
	R: GAGACAGCCAGGAGAAATCAA	
CASP3	F: GAGCCATGGTGAAGAAGGAATA	162
	R: TCAATGCCACAGTCCAGTTC	
CASP7	F: CGAAACGGAACAGACAAAGATG	169
	R: TTAAGAGGATGCAGGCGAAG	
CASP8	F: GCCCAAACTTCACAGCATTAG	160
	R: GTGGTCCATGAGTTGGTAGATT	
CASP9	F: CGACCTGACTGCCAAGAAA	153
	R: CATCCATCTGTGCCGTAGAC	
CYCS	F: GGAGAGGATACACTGATGGAGTA	102
	R: GTCTGCCCTTTCTTCCTTCTT	
P53	F: GAGATGTTCCGAGAGCTGAATG	129
	R: TTTATGGCGGAGGTAGACT	
CYCA	F: TATCTGCACTGCCAAGACTGAGTG	126
	R: CTTCTTGCTGGTCTTGCCATTCC	
ACTB	F: TGGCTGGGGTGTTGAAGGTCT	179
	R: AGCACGGCATCGTCACCAACT	

2.5. cDNA Synthesis and qRT-PCR Analysis

cDNAs were synthesized from total RNAs using the protocol of the manufacturer. The expression levels of genes (BAX, BCL2, CASP3, CASP7, CASP8, CASP9, CYCS and P53) which have an important role in apoptosis and housekeeping genes (ACTB and CYCA), were determined by quantitative Real-Time PCR (qRT-PCR). SyberGreen-I mix was completed with 1X in PCR mixes, 5 pMol forward and 5 pMol reverse primers (Table 1), 2 µl cDNA as template and sterile ddH_2O with a total volume of 20 µl.

The mix was placed onto a RT-PCR platform (Bio-Rad CFX Connect Real-Time PCR System), the heat profile of the reaction was set to +95 ˚C for 10 minutes, 40 cycles (95 ˚C 30 seconds, 60 ˚C 30 seconds, 72 ˚C 30 seconds). Later, melting curve analysis was performed by heating the temperature at 95 ˚C for 1 minute and decreasing the temperature to 55 ˚C gradually until 95 ˚C and by making optical measurements at every 0.2 ˚C increase. The products obtained from RT-PCRs were carried out on a 2% agarose gel and the band sizes were observed to verify that it was the right product.

2.6. Statistical Analysis

qRT-PCR expression data of candidate genes were normalized with housekeeping gene (CYCA and ACTB) data. Volcano Plot analysis was performed with the web-based program called "RT² Profiler™ PCR Array Data Analysis" with the $2^{-(\Delta\Delta Ct)}$ method for the statistical analysis of the quantitation values of the genes. Comparison of the control and dose groups was statistically evaluated by the "Student *t*-test" analysis in the "RT² Profiler™ PCR Array Data Analysis" program.

3. RESULTS and DISCUSSION

3.1. Cytotoxic Effect of Carvacrol in PANC-1 Cells

In this study, the LNCaP cells were cultured under suitable conditions (Figure 1). The cytotoxic effect of *V. opulus* on cancer cells was determined by XTT analysis. The IC_{50} dose was determined to be 100 µg in 48 hours after *V. opulus* application on LNCaP cells (Figure 2). A previous study using an experimental rat colon cancer model reported that gilaburu juice could prevent the progression of established tumors (Ulger *et al*., 2013). Sarıözkan *et al*. (2017) conducted a study to investigate the protective effect of chemotherapy against side effects. It was shown that increasing testicular and sperm injuries were alleviated by *V. opulus* treatment in adult rats treated with docetaxel (DTX) and paclitaxel (PTX) therapy (Sarıözkan *et al*., 2017). In another study using HT29 and SW480 colon cancer cell lines, *V. opulus* inhibited cancer development compared to normal colon epithelial cells (Chojnacka *et al*., 2019). In this study, cytotoxicity tests indicated that *V. opulus* decreased cell proliferation and caused cell death. Our results agree with the findings of the previous literature. Half of the prostate cancer cells died at a dose of 100 µg/mL at 48 hours. It has been reported that *V. opulus* increases cellular cytotoxicity in breast cancer and cervical cancer cell lines, as well as reduces migration in tumor cells. The same study also suggested that *V. opulus*, which has a high antioxidant capacity, could be used as a preservative in high-risk individuals as a food supplement or a pharmacological product (Zakłos-szyda and Pawlik, 2019).

The expression levels of apoptotic genes were examined based on the detected IC_{50} dose (100 µg/mL) using qRT-PCR analysis. The resulting PCR products were electrophoresed on an agarose gel (Figure 3). Also melting curve analysis (data not shown) images indicated that these primer pairs and PCR conditions can be used to evaluate gene expression levels. The fold change expression levels of apoptotic genes were given in Figure 4. The results showed that BAX, CASP7, CASP8 and P53 expression levels were significantly increased. CASP3, CASP9 and CYCS levels were down regulated; however, only steady the state-level of CASP3 gene was found to be significant ($p<0.05$).

Figure 1. Microscopic image of LNCaP human prostate cancer cells (20x).

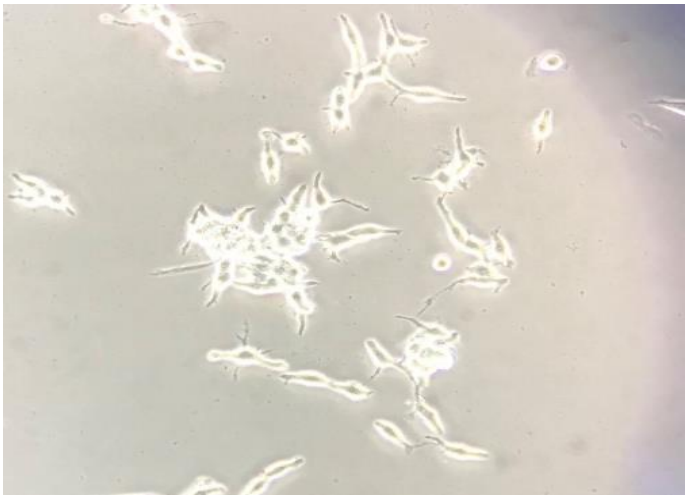


Figure 2. Effects of V. opulus on LNCaP cell line viability at 48 h. IC_{50} value of *V. opulus* was determined as 100 µg/mL at 48 hours.

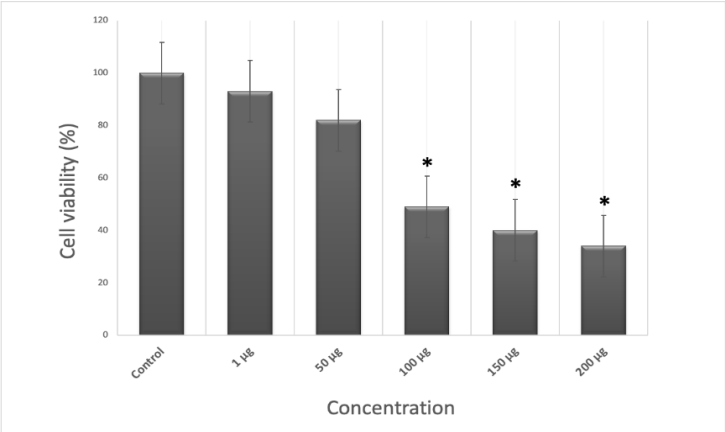
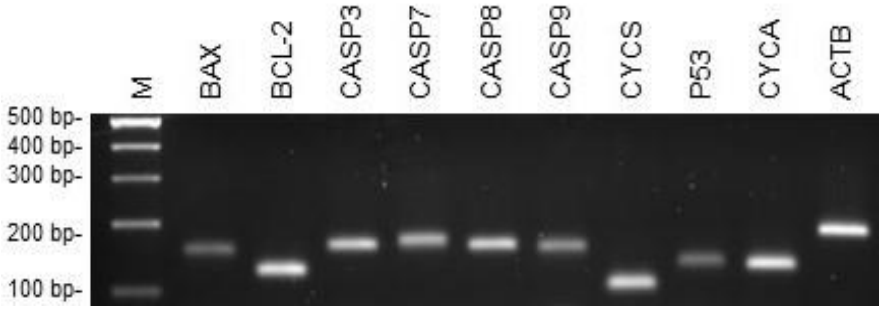
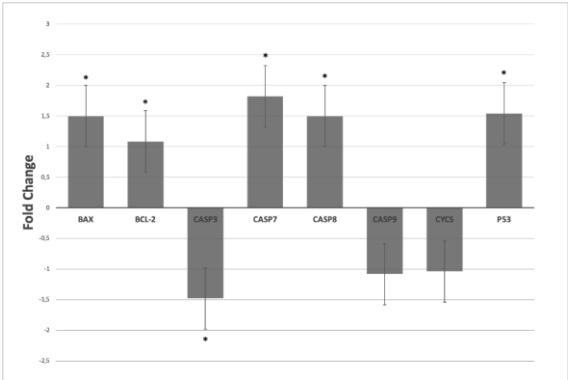


Figure 3. Agarose gel image of resulting RT-PCR products (M: 100 bp size standard). All target and reference genes specifically amplified corresponding genomic region.



The significant increase of BAX, CASP7, CASP8 and P53 expressions indicates activation of the proapoptotic pathway. The significant downregulation in CASP3 expression suggests that CASP7 may be effective in the external pathway instead of CASP3. Apart from these, the decrease in cell viability may have occurred through different death mechanisms. This will be an idea for further studies to investigate the autophagy pathway, which may explain the decrease in cell viability after applying *V. opulus*. Although *in vitro* application illustrated a cytotoxic effect on LNCaP cells, there is a need for further *in vivo* studies to investigate the molecular mechanism of anticancer effects of *V. opulus*.

Figure 4. RT-PCR analysis of apoptosis-associated genes. * indicates $p < 0.05$. Expression levels of target genes were normalized with reference genes, and $2^{-(\Delta\Delta Ct)}$ values were compared with the control group.



4. CONCLUSION

The main target of drug regimens used in cancer treatment is to ensure the death of cancer cells with specific mechanisms. Many chemotherapeutic agents are also developed for this purpose. However, any non-targeted treatment is a risk factor for healthy cells. Some herbal chemicals show natural anti-cancer effects. Their use also depends on adequate clinical studies of the active ingredient; plants contain several active substances simultaneously. *V. opulus* is a plant that is consumed in local regions as both nuts and fruit juice. Besides many known clinical benefits, the potential anti-cancer effect is also illustrated in here.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Omer Gok: Study design, conducting experiments, statistical analysis, evaluation of the results, preparation of the manuscript. **Fatma Secer Celik:** Study design, conducting experiments, statistical analysis, preparation of the manuscript. **Canan Eroglu Gunes:** Study design, statistical analysis, preparation of the manuscript. **Ercan Kurar:** Statistical analysis, preparation of the manuscript.

Orcid

Omer Gok https://orcid.org/0000-0002-1200-3597
Fatma Secer Celik https://orcid.org/0000-0001-5619-8958
Canan Eroglu Gunes https://orcid.org/0000-0002-3796-575X
Ercan Kurar https://orcid.org/0000-0002-9234-1560

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Status of Bacterial Entomopathogens Used for Microbial Control of Arthropod Pests in Iran and Turkey

Rasoul Marzban [1*], Gozde Busra Eroglu [2]

[1] Biological Control Research Department, Iranian Research Institute of Plant Protection, Agricultural Research Education and Extension Organization (AREEO), Tehran, Iran
[2] Department of Molecular Biology and Genetics, Faculty of Science, Erzurum Technical University, Erzurum, Turkey

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Abstract

Bacterial entomopathogens, especially *Bacillus thuringiensis* (*Bt*), are the most popular entomopathogen in bacteria worldwide, thanks to their toxin genes and high virulence. In this context, to develop an alternative to chemical pesticides used to control agricultural and forest pest insects, *Bt* has been isolated from soils and many insects, and products in the form of formulations have been prepared. It is also known that *Bt*-based biopesticides are generally more specific and harmless to other organisms. The results demonstrate that countries have different conditions for releasing these organic products to the market and it is often difficult for scientists to obtain a license. This study discusses the current status of bacterial entomopathogens study in Iran and Turkey.

Corresponding Author:
Rasoul Marzban, Biological Control Research Department, Iranian Research Institute of Plant Protection, Agricultural Research Education and Extension Organization (AREEO), Tehran, Iran, r.marzban@areeo.ac.ir
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1. INTRODUCTION

Agricultural and forest pest insects cause economic losses of millions of dollars every year. Chemical pesticides are the leading strategies used to control these pests because of their short duration of action and ease of use. However, the broad spectrum of action of chemical pesticides destroys non-target organisms such as beneficial insects and natural enemies of destructive pests. These products are toxic to both the environment and humans and cause resistance development in target organisms over time (Karimi *et al*., 2019; Khan *et al*., 2023).

Another strategy involves the use of formulations of natural pathogen origin to protect agricultural crops from invaders such as pests, fungi, and weeds. These products are effective for the target organism and provide safe use for other living creatures and nature (Egbuna *et al*., 2020). In recent years, more than 150 isolates of *Bacillus thuringiensis* (*Bt*) isolated from cultivated soils, were tested on different groups of insect pests after purification. In addition, some research has been carried out to determine the efficacy of those native isolates to select the ideal isolate (Marzban and Salehi, 2006). The diversity of the isolates *cry* and *cyt* genes have been studied (Salehi *et al.,* 2007; Salehi *et al.,* 2008; Seifi Nejad *et al.,* 2008; Nazarian Amirani *et al*., 2009; Chalajour *et al.,* 2013; Rashki *et al*., 2021). Moazami started to study on production and formulation of insect bacterial pathogens, especially *B. thuringiensis israelensis* H14 several decades ago. These studies are continuing and work is already well done on mass production and formulation of *Bt* in the Iranian research institute of plant protection and Agricultural biotechnology research institute (Marzban, 2012; Khorramvatan *et al.,* 2013; Khorramvatan *et al.,* 2014; Marzban *et al.,* 2014; Saberi *et al.,* 2014; Salehi *et al.,* 2015; Marzban *et al.,* 2016). With the increasing interest in organic agriculture in recent years, the

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decrease in the use of chemical pesticides and the development of *Bt*-based microbial products instead is a tremendous development for the pesticide market.

Since the 1960s, biopesticides have been tested to control pests on wood trees and fruit gardens in Iran. The first biopesticide based on *B. thuringiensis* was successfully used against Gypsy moth in wood trees (Reardon *et al*., 1994). During the past three decades, many research activities have been conducted on entomopathogens and antagonistic agents, such as fungi, bacteria, viruses, and nematodes. Furthermore, numerous efforts have been performed to obtain some biopesticide products supported by the Iranian Government and private companies at a commercial level (Karimi *et al*., 2019).

In Turkey, many entomopathogenic bacteria have been isolated from different insects for about 40 years, and their use as a biological control agent has been investigated. The first trial of *B. thuringiensis* product with Turkish isolates was studied by Çakmakcı *et al.,* (1985) with the TUBITAK-Tarmik-3 project. In this project, *B. thuringiensis* was isolated from died *Hyponomeuta malinellus* larvae collected from Kütahya, Niğde, and Ankara. The formulations of these strains and two commercial products (Dipel and Thuricide) were compared *in vitro* against the larvae of *Ephestia kuehniella*, *Galleria mellonella*, *Amorphogynia necessaria*, and *Malacosoma neustria*. The most effective local *Bt* strain was selected and field trials were conducted in Ankara. Studies in field trials were carried out on both *H. malinellus* and beneficial insects in the region. Özkazanç (1986) used different doses of Tarmik-3 *Bt* for the control of *Thaumetopoea pityocampa* and reported that the mortality rate was between 40-99% as a result of this study. Alten and Boşgelmez (1990) reported that 2 *Bt* (Iskenderun-310109 and Icel-330218) and 2 *Bacillus sphaericus* (Iskenderun-310111 and Icel-3302120) isolates of Turkish origin have high virulence for the control of 2nd and 3rd instar larvae of *Culiseta longiareolata*. Çakmakçı *et al.,* (1990) investigated the toxicity of *B. thuringiensis* subsp. *israelensis* (number 330211) isolated from the İçel region of Turkey to mammals on mice and guinea pigs. In this study, $2x10^8$ bacterial stock suspensions per mL were given to the experimental animals by different means such as subcutaneous intraperitoneal, intravenous, oral, percutaneous, inhalation, and eye irritation test for each vaccination, and no signs of disease were observed in any of the animals as a result of the experiment. Sezen and Demirbağ (1999) were isolated a *B. thuringiensis* subsp. *kurstaki* from hazelnut pest, *Balaninus nucum* larvae, and adult in Trabzon. In insecticidal activity studies using this isolate, 100% mortality was detected on *Agriotes lineatus* at 1.8×10^9 cfu/mL concentration (Danışmazoğlu *et al.,* 2012). Besides, *Bt* isolate was applied to *Spodoptera littoralis* and *Agelastica alni* and 100% and 72.3% mortality were observed, respectively (Çakıcı *et al.,* 2014; Eski *et al.,* 2017). Sezen *et al.,* (2008) isolated *B. thuringiensis* ssp. *tenebrionis* (Xd3) from *Xyleborus dispar*. This isolate was applied to different hosts (*Agelastica alni*, *Amphimallon solstitiale*, and *Melolontha melolontha*) at a concentration of 1.8×10^9 cfu/mL, and 100% mortality was observed in all of them. Katı *et al.,* (2009) isolated two *B. thuringiensis* subsp. *morrisoni* from *Thaumetopoea pityocampa* in Turkey. As a result of virulence tests, Tp6 isolate caused 100% mortality in *Agelastica alni* and *Leptinotarsa decemlineata* larvae, while Tp14 isolate caused 100% mortality in *Malacosoma neustria* larvae. Yılmaz *et al.,* (2012) investigated the insecticidal activity of *Bt* SY49.1 strain in their study on *Ephestia kuehniella* and *Plodia interpunctella* and reported that spore-crystal mixtures caused more than 90% mortality in the larvae of the pests. Yıldız and Sezen (2017) isolated 13 isolates of *Serratia marcescens* (Eca1 and Eca3), *Serratia* sp. (Eca 11), *B. thuringiensis* (Eca2, Eca4, Eca6-10, Eca12 and Eca13), and *B. thuringiensis* (Eca5) from *Cadra cautella*. The insecticidal activities of these isolates were performed against three insect species from the Lepidoptera group that caused serious damage in warehouses. The highest insecticidal activity was 57% for Eca9 isolate in 3rd instar larvae of *C. cautella*, 100% for Eca9 isolate in 3rd instar larvae of *Plodia interpunctella*, and 100% for Eca10 and Eca3 isolate in 3rd instar larvae of *E. kuehniella*. Accordingly, they reported that Eca9, Eca3, and Eca10 isolates could

be valuable as potential biological control agents for the control of warehouse pests. Eski *et al.,* (2018) *Bt* (Se13 strain) isolated from *Spodoptera exigua*, at a concentration of 1.8×10^9 cfu/mL, caused 75% mortality 48 hours after the application and 100% mortality after 7 days in the 3rd instar larvae of *S. exigua*. Eski *et al.,* (2019) microencapsulated a local *Bt* Se13 isolate to make it environmentally stable. As a result of the efficacy studies of this product on *Spodoptera exigua*, the LC_{50} value was determined as $1.6x10^4$ cfu/mL^{-1} and they reported that this biopesticide is promising for field applications of *S. exigua* control. Eski *et al.,* (2022), reported that *Bt* Xd3 against *Leptinotarsa decemlineata* showed 83% and 73% mortality in larvae and adults, respectively, within 10 days when applied at 10^9 cfu/mL^{-1}concentration. Usta (2022), *Bt* from *Cydalima perspectalis* collected from Artvin province and reported that this bacterium showed an 85% mortality rate on the pest. Eski (2023) tested 13 local *Bt* strains isolated from soil samples for the control of *Tuta absoluta* and declared that *Bt*-B3 isolate is a promising biological warfare agent for integrated pest control of *T. absoluta* in Turkey.

1.1. Application of Biological Control Agents

In Iran, although the main approach to pest control in the country has been dominantly chemical control for years, biological pest control with about 90 years of practical background has occupied a meaningful part of pest control programs. It started with the classical approach in 1933 by introducing the Australian ladybird, *Rodolia* (=*Novious*=*Vedalia*) *cardinalis* to control an exotic pest, Australian scale, *Icerya purchasi*, on citrus. Afterward, other natural enemies such as *Cryptolaemus montrouzieri*, *Prospaltella berlesei* and *Phytoseiulus persimilis* were introduced to the country (Karimi and Kamali, 2021). The augmentation method was also practiced in 1946 by augmenting sun pest egg parasitoids and releasing them on infested wheat which lasted for 2 decades. From a conservation point of view, there is an outstanding example in the history of biological control. In 1972, sugarcane stem borer broke out and chemical control failed in suppressing the pest outraged population. The idea of banning chemical application made the natural enemy (*Platytelenomus hylas*) escapes from chemical pressures and boosts its population which resulted in a sustainable pest control strategy (Karimi and Madadi, 2021). At present, about 100 private companies are involved in the biological control industry. Natural enemies such as *Trichogramma* sp., *Habrobracon hebetor*, *Chrysoperla carnea,* and *Cryptolaemus montrouzieri* are masse produced by private companies under close monitoring of Plant Protection Organization and released in selected farms based on protocols provided by the research section. Moreover, microbial biological control agents based on *B. thuringiensis*, *B. subtilis, Trichoderma* sp., are finding their role in sound crop protection. Since the 1970s, biopesticides have been tested to control pests on wood trees and fruit gardens in Iran. The first biopesticide based on *B. thuringiensis* was successfully used against gypsy moth in wood trees (Karimi *et al*., 2019). During the past three decades, many research activities have been conducted on entomopathogens and antagonistic agents, such as fungi, bacteria, viruses, and nematodes. Furthermore, numerous efforts have been performed to obtain some biopesticide products supported by the government and private companies at the commercial level.

Biological control studies in Turkey started in the Ottoman period. In those years, mainly beneficial insects were procured from abroad and released into nature, and pest populations were controlled. The first example of this was provided in 1910 by bringing the predator *Rodolia cardinalis* from Chios to control the *Icerya purchasi* species, which is harmful in citrus orchards and some fruits (İslamoğlu, 2021). In 1965, Antalya Biological Control Research Station was established and applications were made here for many years in the form of reproduction and release of predator and parasitoid species (Erkılıç and Demirbaş, 2007).

The isolation and use of local microbial pathogens in biological control started in Turkey towards the end of the 1980s. So far, many local isolates of bacterial, fungal, nematode,

protozoan, and virus origin have been obtained by researchers. Most of these isolates were biotested under laboratory conditions, their host spectra were determined, and pot and field trials were completed. Among the microbial pathogens, studies on *Bt* isolates stand out in Turkey. Thanks to the toxin genes of *Bt* isolate, both its virulence and its host spectrum are relatively broad, this has made it possible to focus on studies in this field. However, although there are many local, national, and effective *Bt* isolates in the research laboratories of our country, commercialization of these products to the market has not been possible until now due to the high cost of the licensing process. For this reason, foreign biopesticide products supplied from abroad are used in Iran and Turkey. However, the high cost of foreign products has pushed agricultural producers to use lower-cost chemical products. However, it is known that the use of products of foreign isolate origin may have a negative effect on natural strains and the susceptibility of the host may vary according to foreign isolates. Therefore, local isolates suitable for growth as microbial control agents should be preferred for the identification of new species present in each geographical region.

1.2. Application of *B. thuringiensis* as Biopesticide

The reason why the use of microbial pesticides is still not widespread is the lack of social awareness; distrust of farmers (Mishra *et al*., 2015); and lack of opportunities to support the use of microbial pesticides (Kumar and Singh, 2015). However, producers who think that biological control alone will not be sufficient for pest control should be given the necessary information and, if necessary, should be directed to integrated control methods. By supporting farmers with an affordable price policy and consistent success of microbial products, the use of products containing microbial agents can be expanded (Marrone, 2009; Marzban and Askari 2010; Regnault-Roger, 2012). *B. thuringiensis* has been used in Iran for three decades. It has been mostly used on agricultural and forestry pests. The product was produced by a private company in semi-solid culture in 1995. However, the product has not been registered due to high levels of microbial contamination (10-20%), beta-exotoxin presence, and unsuitable formulation. Later, the bacterium was produced using the liquid fermentation method in two private companies, Biorun Co. and Mehr Asia Biotechnology Company. The used serotypes of *B. thuringlensis* consist of *kurstaki*, *israelensis*, and *aizawai*. The current market for *Bt*-based products in Iran is approximately $400.000 sharing only 1.0% of the total market. The most important Bt crop used in Iran is *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* H14 and is commercialized for the control of many pests. The efficiency of biopesticides based on *B. thuringiensis* has been proved on pests of forestry, tropical crops, vegetables, industrial plants, orchards, and fruits in both glasshouse and field conditions. The target pests are gypsy moth, *Lymantria dispar;* garlic worm, *Dyspessa ulula*, indian meal moth, *Plodia interpunctella,* sugar beet armyworms, *Spodoptera littoralis, Spodoptera exigua;* cabbage butterfly, *Pieris brassicae, Plutella xylostella;* potato tuber moth, *Phthorimaea operculella;* grape berry moth, *Lobesia botrana;* european corn stem borer, *Ostrinia nubilalis*; colorado potato beetle, *Leptinotarsa decemlineata;* apple mine moth, *Yponomeuta malinellus;* rice stem borer, *Chilo suppressalis;* pea bollworm, *Helicoverpa viriplaca; Agrotis* spp.; oak white moth, *Leucoma wiltshire;* cotton bollworm, *Helicoverpa armigera;* mosquito, *Anopheles* sp. In recent years, more than 30 tons per year of *B. thuringiensis* products have been used for controlling lepidopteran pests in the forest, tropical crops, vegetables, industrial plants, orchards, and fruit orchards. In addition, some research has been carried out to explore native isolates to determine their efficacy and screen the ideal isolate for the control of coleopteran pests. (Saberi, *et. al*., 2020; Saberi, *et. al*., 2023)

B. thuringiensis israelensis H14 strain has high virulence in Diptera species (mosquito and housefly larvae) (De Barjac and Sutherland, 2012). Various studies have shown that *B. thuringiensis* H-14 is more effective against *Culex* and *Aedes* than *Anopheles* mosquitoes

(Moazami, 2002). *B. thuringiensis* H-14, which is known to be harmless to humans, pets, fish or plants, has been produced as a formulation for over ten years. There are 10 *Bt* based products registered and used between 1996-2021 in Turkey. Since all of these products are imported, they are quite expensive. This situation leads to the continuation of the intensive use of chemical pesticides in Turkey. For this reason, there is a need to increase the support for the introduction of local biopesticides produced in research laboratories of universities to the market with the support of companies to reduce the foreign dependency of the country and to expand the use of environmentally friendly products. Thus, it will contribute to both the economic and health security of Turkey.

1.3. Quality Control and Registration of Microbial Biopesticides

In order to commercialize biopesticide products so that they can compete with chemical pesticides, the required tests need to be carried out more easily. Otherwise, the development and commercialization of microbial biopesticides is a very difficult and time-consuming process. The Iran Agricultural Research, Education, and Extension Organization is improving and developing biopesticides for use in sustainable development. In 1994, the High Council for Development of the Application of Biologic Substances and Optimized Use of Fertilizer and Pesticides in Agriculture was established at the Ministry of Agriculture. The council's main objective is to reduce pesticide use by 30%. The government has also decreased subsidies for pesticides and is proceeding with a full omission of pesticides. The government provides credits and bank loans for the production of biopesticides In addition, the production of biopesticides is exempted from a series of taxes and duties. One of the most important issues in turning *Bt*-based insecticides into a commercial product is that the product maintains its stability. While most manufacturers tested this with live spore counting in the 1980s, the correlation between spore count and toxicity later revealed that this test was unreliable. Thus, in 2010, the quality control scheme for biopesticides was revised by the Plant Protection Research Institute of Iran and the Plant Protection Organization (Marzban, 2004).

A committee within the Ministry of Agriculture in Iran has the authority to register pesticides for use. All produced batches of *Bt* must be assayed for quality control. This quality control includes contamination with other microorganisms, β-exotoxin, suspensibility, potency, and viable spore count assay.

Turkey is in a very important position in terms of climate diversity, having large agroecological areas and growing many products of economic importance. In recent years, the number of producers engaged in organic farming has increased with government support. According to the revised directive published in the Official Gazette No. 27347 on 12.9.2009, under the Plant Protection and Quarantine Law No. 6968, registration procedures are carried out by the Ministry of Agriculture and the General Directorate of Rural Affairs Protection and Control. Since the criteria used in the inspection process of microbial pesticides are the same as the standards used for chemical pesticides, there are difficulties in the licensing process. For this reason, making regulations in the regulations of license and safety tests will make it easier for scientists to put their products on the market. Thus, by providing both lower-cost and more reliable products, the persuasion process of agricultural producers on the use of biopesticides will be facilitated. There are 16 registered microbial agent-based preparations in Iran (Table 1).

Table 1. Microbial biopesticides registered in Iran (Iranian Plant Protection Organization)

Product name	Microorganism type	Active ingredient	Provider	Approval date	Formulation	Manufacturer/ Country	Target pest	Crop
CangMei		*Bacillus subtilis*	Giyah Parnian Atlas	2018	WP	Deqiang Biology /China	*Pyricularia oryzae*	Rice
				2022	WP		*Alternaria* spp.	Tomato
Pars Bacill		*Bacillus velezensis*	Royan Tisan Sabz	2019	SC	Royan Tisan Sabz/Iran	*Fusarium oxysporum*	Greenhouse tomato
Talaromin		*Talaromyces flavus*	Baharan Dasht Sahel	2018	P	Baharan Dasht Sahel/Iran	*Verticillium dahliae*	Potato
Trianum- P				2012	WP		*Fusarium oxysporum*	Greenhouse tomato
			Giyah	2012	WP	Koppert /Netherlands	*Fusarium oxysporum*	Greenhouse cucumber
Trianum- G		*Trichoderma harzianum*		2018	G		*Fusarium oxysporum*	Cantaloupe
	Fungal/fungus like			2018	G		*Fusarium oxysporum*	Greenhouse tomato
Serenade			Bayer Parsian	2019	SC	Bayer/ Germany	*Botrytis cinerea*	Greenhouse strawberry
		Bacillus subtilis		2021	SC		*Fusarium oxysporum*	Greenhouse tomato
Rooein-1			Zist Fanavar Sabz	2020	P	Zist Fanavar Sabz/Iran	*Rhizoctonia solani*	Sugar beet
Polyversum		*Pythium oligandrum*	Bazargan Kala	2021	WP	Biopreparaty/ Czech Republic	*Pythium* spp.	Greenhouse cucumber
Naturalis-L		*Beauveria bassiana*	-	2001	SC	CBC/Italy	*Bemisia tabaci*	Cotton
Mycotal		*Lecanicillium muscarium*	Giyah	2012	WP	Koppert/ Netherlands	*Trialeurodes vaporariorum*	Greenhouse tomato
Biolep				2018	SC		*Lobesia botrana*	Grape
			Biorun	2018	SC	Biorun/Iran	*Plutella xylostella*	Cabbage
Biolep-p	Bacterial			2018	WP		*Plutella xylostella*	Cabbage
		B. thuringiensis		2019	WP		*Helicoverpa armigera*	Cotton
Bactospeine			Valnet Bioscience	1968	WP	Valnet Bioscience/USA	*Lymantria dispar*	Forrest trees
Bt			Giyah Parnian atlas	2022	WP	Biontech International/India	*Heliothis viriplaca*	Pea
MVP			Ecogen/USA	1975	WP	Ecogen/USA	*Leucoma wiltshire*	Forrest trees
Capsanem	Nematode	*Steinernema carpocapsae*	Giyah	2021	P	Koppert/ Netherlands	*Phthorimaea operculella*	Stored Potatoes

1.4. Registered Microbial Biopesticides

It is known that 74% of biopesticides, which have a market share of 5.61 billion USD in 2021, are bacteria-based (Thakore, 2006). Since the 1960s, biopesticides have been tested to control pests on tree trees and orchards in Iran. The first biopesticide based on *B. thuringiensis* was successfully used against the gypsy moth on woody trees. In the last three decades, many research activities have been conducted on entomopathogens and antagonist agents such as fungi, bacteria, viruses, and nematodes. In addition, many studies have been carried out to obtain some biopesticide products that are commercially supported by the Iranian government and private companies. *B. thuringiensis*-based products account for the largest share in the Iranian microbial biopesticide market. These products were first registered in Iran 50 years ago (under the trade names MVP® and Bactospin® on December 22, 1968 and May 9, 1975, respectively). Apart from *Bt*, other microbial products in the Iranian market are of fungal origin. On this subject, many researchers in Iran are working on microbial biocontrol agents against the problems of different plant pests (Moazami, 2002; Marzban and Tajbakhsh, 2004; Ranjy *et al*., 2005; Keshavarzi, 2008; Seifi Nejad *et al*., 2008; Nazarian Amirani *et al*., 2009; Shojaaddini *et al*., 2010; Marzban, 2012; Marzban *et al*., 2014; Saberi *et al*., 2014; Marzban *et al*., 2016). According to the Turkish Statistical Institute, vegetable production in Turkey increased by 1.8 percent in 2021 compared to the previous year and reached 31.8 million tons. Rooted and tuberous vegetable production increased by 6.9 percent in vegetable subgroups. In this direction, Turkey's need for insecticides in agricultural production is also increasing. There are 29 registered microbial agent-based preparations (15 bacterial, 13 fungal, and 1 viral) in Turkey (Table 2).

Table 2. Microbial biopesticides registered in Turkey (Turkey Plant Protection Organization)

Product name	Microorganism type	Active ingredient	Provider	Approval date	Formulation type	Manufacturer/Country	Target pest	Crop
Agree 50 WG			Gennova	1996	WG	Certis/USA	*Helicoverpa armigera, ...*	Tomato
BIO-T Plus			Cansa	2009	Liquid	Biodalia Microbiologibal Technologies/Israel	*Lobesia botrana, ...*	Red pine, …
Dacron			Safa	2012	Wettable powder	Jiangsu International Cooperation/China	*Cryptoblabes gnidiella, Helicoverpa armigera*	Orange, tomato
Delfin	Bacterial	*B. thuringiensis*	Agrikem	1996	WG	Certis/USA	*Tuta absoluta, ...*	Tomato, ../
Dipel			Sumitomo	2010	WG	Valent Biosciences/ABD	*Spodoptera littoralis, ...*	Tobacco,...
Florbac			Sumitomo	2010	WG	Valent Biosciences/ABD	*Spodoptera littoralis, ...*	Tobacco, …
Foray			Envirotek	2008	Liquid	Valent Biosciences/ABD	*Thaumetopoea pityocampa, ...*	Pine trees
IAB-BT			Nektar	2010	WG	Investigaciones Y Aplicaciones Bıo Tecnologicas/Spain	*Helicoverpa armigera, ...*	Apple, …
Rapax			Imes	2009	Liquid	CBC (Europe) S.r.l./Italy	*Helicoverpa armigera, ...*	Vineyard, tomato
Rebound			Hektaş	2005	Wettable powder	Shandong Lukang Biological Pesticides/China	*Tuta absoluta, , ...*	Vineyard, …

Subtilex foliar		*Bacillus subtilis*	Bioglobal	2010	Wettable powder	Becker/ABD	*Botrytis cinerea*	Vineyard, tomato
Serenade			Bayer	2004	Liquid	Bayer/Germany	*Botrytis cinerea,...*	Tomato, …
Companion			Hekagro	2011	Liquid	Growth products/ABD	*Venturia inaequalis,...*	Pear, cherry, …
Biobac-WP			Atlantik	2008	Wettable powder	Biotech/Taiwan	*Sclerotinia sclerotiorum*, , ...	Cherry, apricot, …
AQ10		*Ampelomyces quisqualis*	Boyut	2010	WG	CBC (Europe) S.r.l./Italy	*Erysiphe necator,*	Tomato
Bio Nematon	Fungal/fungus like	*Paecilomyces lilacinus*	Agrobest	2011	Liquid	T. Stanes and Company Limited/India	*Meloidogyne* spp.	Potatoes, …
Bioact		*Paecilomyces lilacinus*	Bayer	2018	DC	Bayer/Germany	*Meloidogyne* spp.	Pepper, eggplant, …
Blossom Protect		*Aureobasidium pullulans*	Nufarm	2012	WG	San Agrow Holding Gmbh/Austria	*Erwinia amylovora*	Pear
Dopteril		*Beauveria bassiana*	Boyut	2009	Liquid	CBC (Europe) S.r.l./Italy	*Bemisia tabaci, ...*	Tomato, …
Nostalgist		*Beauveria bassiana*	Agrobest	2012	Liquid	T. Stanes and Company Limited/India	*Melolontha melolontha, ...*	Cotton,...
Inferno		*Myrotheciu m verrucaria*	AMC-TR	2010	-	-	*Meloidogyne* spp.	Tomato
Nibortem		*Verticillium lecanii*	Agrobest	2012	Liquid	T. Stanes and Company Limited/India	*Frankliniella occidentalis,*	Cucumber
Nogall		*Agrobacterium radiobacter*	Bioglobal	2005	Wettable powder	Becker Underwood/Australia	*Agrobacterium tumefaciens*	Cherry, peach
Remedier		*Trichoderma asperellum*	Tancan	2010	Wettable powder	Isagro S.P.A/Italy	*Alternaria spp., ...*	Strawberry, …
Ruotshield			Hasel	2009	Granular	Biowork/ABD	*Rhizoctonia solani,...*	Cucumber
T-22 Planter Box			Hasel	1999	Wettable powder	Biowork/ABD	*Botrytis cinerea, .,...*	Tomato, cotton
Trichoflow		*Trichoderma harzianum*	Enerji	2002	Wettable powder	McHort/New Zealand	*Fusarium* spp,...	Tomato
Priority		*Paecilomyces fumosoroseus*	Agrobest	2011	Liquid	T. Stanes and Company Limited/India	*Panonychus ulmi, ...*	Apple,...
Madex	Viral	*Cydia pomonella granulovirus*	Verim	2004	Liquid	Andermatt/Switzerland	*Grapholita funebrana*	Plum

Important studies on microbial control factors have been carried out in Turkey for the last 40 years. However, most of these studies remained in the literature and could not be commercialized. In this direction, producers of organic farming prefer imported products because they cannot reach ready-to-use domestic and national biological control agents. As a result, due to the expensiveness of these imported microbial pesticides, the orientation of the producer is generally towards the use of chemical pesticides.

4. CONCLUSION

Chemical pesticides, which are commonly used to control agricultural pests, pose significant health risks to living organisms. In particular, for natural enemies of insect pests using the same product over and over again for all pests both cause resistance in insects and

harm the environment. For this reason, it is necessary to increase the use of natural enemies and to introduce cheap, domestic, and organic alternative products to the market. *B. thuringiensis* species, which is one of the microbial control agents, has a high infectivity in insects, so there are many studies in the literature on this subject. However, since the licensing process of each country has different difficulties, scientists cannot offer the pathogens they have determined to be effective in the laboratory as a product to the markets. After 2000, Turkey and Iran registered at least one microbial pesticide per year, despite a delayed start. The most important reason for less development of microbial pesticides in Iran and Turkey is their expense and comparing their efficiency with chemical pesticides. As a result, producers should be informed about the use of microbial pesticides and product costs should be offered to producers at more affordable prices with government support.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in NatProBiotech belongs to the author(s).

Author Contribution Statement

Rasoul Marzban: Study conception and design, data collection, analysis and interpretation of results, draft manuscript preparation. **Gozde Busra Eroglu:** Data collection, analysis and interpretation of results, draft manuscript preparation.

Orcid

Rasoul Marzban https://orcid.org/0000-0001-5091-006X
Gozde Busra Eroglu https://orcid.org/0000-0001-8988-1315

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